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1,25 Dihydroxyvitamin D_3 Activates Sphingomyelin Turnover in ROS17/2.8 Osteosarcoma Cells without Sphingolipid-Induced Changes in Cytosolic Ca^{2+}

Riting Liu,* Yihuan Xu,* Mary C. Farach-Carson,* James J. Vogel,† and Norman J. Karin†
*Department of Biological Sciences, University of Delaware, Newark, Delaware; and
†Department of Basic Sciences, University of Texas Dental Branch, Houston, Texas

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1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] initiates the hydrolysis of sphingomyelin in ROS 17/2.8 osteosarcoma cells with the resultant generation of cell-associated ceramide. Increases in ceramide levels were detectable at 15 min and maximal one hour after exposure of cells to 1,25$(OH)_2D_3$. Neither 1,25$(OH)_2D_3$ nor exogenous ceramide elicited a change in cytosolic free Ca^{2+} ($[Ca^{2+}]_i$). Transient elevations in $[Ca^{2+}]_i$ were observed when cells were exposed to exogenous sphingosine, but there was no detectable conversion of ceramide to sphingosine in 1,25$(OH)_2D_3$-treated cells. Ceramide also did not stimulate Ca^{2+} uptake across ROS 17/2.8 cell plasma membranes. Collectively, these results suggest that 1,25$(OH)_2D_3$ activates sphingomyelin turnover in ROS 17/2.8 osteosarcoma cells but that the sphingolipid metabolite ceramide is not responsible for 1,25$(OH)_2D_3$-induced activation of plasma membrane Ca^{2+} channels. © 2000 Academic Press

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1,25$(OH)_2D_3$ acts on target cells via both long-term nuclear receptor-mediated and rapid membrane-initiated pathways. These pathways account for the pleiotropic effects of 1,25$(OH)_2D_3$ on osteoblasts and a variety of other cells (reviewed in 1, 2). Rapid actions of 1,25$(OH)_2D_3$ include Ca^{2+} influx via L-type voltage-sensitive Ca^{2+} channels (VSCCs) in the plasma membrane of osteoblasts (3, 4), modulation of membrane receptor-mediated protein kinase in growth plate chondrocytes (5), rapid Ca^{2+} movement across intestinal epithelial cells (6), intracellular Ca^{2+} increases and protein kinase C translocation in keratinocytes (7, 8), and release of Ca^{2+} from intracellular stores (9, 10). Recent studies showed that modulation by 1,25$(OH)_2D_3$ of the phosphorylation of the extracellular matrix protein osteopontin depends on the membrane-initiated Ca^{2+} influx (11).

Recent studies on the mechanism of 1,25$(OH)_2D_3$ action suggest a role for the sphingolipid signaling pathway. Ceramide, a product of sphingomyelin hydrolysis, can serve as a second messenger to mediate the effects of a number of effectors including 1,25$(OH)_2D_3$ (ref. 12), tumor necrosis factor-α (13, 14), γ-interferon (13), interleukin-1β (15, 16), nerve growth factor (17), dexamethasone (18), oxidized low density lipoprotein (19), polyunsaturated fatty acids (20), as well as anti-CD28 and anti-Fas antibodies (21, 22). Ceramide was first proposed as a second messenger when it was found to mediate the effects of 1,25$(OH)_2D_3$ on the differentiation of HL-60 promyelocytic cells (12, 23). The time course for ceramide generation varies from seconds to hours among different cell types. Ionizing radiation (24), antibodies against Fas receptor (22), interleukin-1β (16), and TNF-α (14) induced changes in intracellular ceramide concentration within seconds or minutes.

The mechanism by which 1,25$(OH)_2D_3$ activates VSCCs is unknown. We previously demonstrated that sphingosine derivatives elicited the release of Ca^{2+} from intracellular stores in pre-osteoblastic cells (25). Here we present the results of experiments designed to determine if 1,25$(OH)_2D_3$ induces sphingomyelin turnover in osteoblastic cells and to ascertain whether this process is linked to 1,25$(OH)_2D_3$-induced Ca^{2+} influx through VSCCs.

MATERIALS AND METHODS

Materials. 1,25$(OH)_2D_3$ was purchased from Calbiochem (La Jolla, CA). L-[3-^{3}H]serine, [1-^{14}C]palmitic acid and Amplify fluorographic reagent were from Amersham Life Science (Arlington Heights, IL). Insulin, transferrin, ceramide, C2-ceramide, C18-ceramide, sphingosine and sphingomyelin were from Sigma (St. Louis, MO). Fura-2/AM was from Molecular Probes, Inc. (Eugene, OR). $^{45}Ca^{2+}$ was from NEN Life Sciences (Boston, MA).

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Cell culture. ROS 17/2.8 rat osteosarcoma cells were grown in Ham's F-12/Dulbecco's Modified Eagle's Medium (1:1) (F-12/DMEM), containing 10% fetal calf serum as described previously (3).

Metabolic labeling of lipids with [^{3}H]serine. ROS 17/2.8 cells (approximately 80% confluence) were washed three times with phosphate buffered saline, then incubated with 5 μM L-[3-^{3}H]serine (5 μCi/ml, specific activity 30 Ci/mmol) for 48 h in serum-free F-12/DMEM medium containing insulin (5 mg/l) and transferrin (5 mg/l) (12, 26). The radioactive medium was removed and cells were washed with serum-free F-12/DMEM. The cells were incubated at 37°C for 20 min, then for the times after addition of 1,25$(OH)_2D_3$ or the same volume of ethanol vehicle.

Incubations were terminated by aspiration of the medium and the cultures immediately were fixed with ice-cold methanol. [1-^{14}C]-palmitic acid (20,000 dpm) was added for quantitation of the extraction efficiency. Extraction of total lipid was performed by the method of Bligh and Dyer (27). Briefly, chloroform/methanol/H_2O (1/2/0.8 v/v/v) was used for extraction followed by separation in chloroform/methanol/H_2O (2/2/1.8 v/v/v). Total lipid in the organic phase was dried under N_2, then resuspended in 100 μl chloroform. For thin layer chromatographic analysis, 20 μl of the sample was applied to silica gel 60 thin-layer chromatography (TLC) plates (Merck, Germany). Ceramide and sphingosine were separated on the plate developed in chloroform/methanol/3N NH_4OH (30/20/0.6 v/v/v); sphingomyelin was resolved in chloroform/methanol/2N NH_4OH (60/35/5 v/v/v). The plate was removed from the solvent when the solvent front had risen to within 1.5 cm of the top of the plate. Lipids were visualized with iodine vapor, scraped and eluted from the silica gel with chloroform/methanol (2:1 v/v). The separated radiolabeled sphingolipids were identified by co-migration with commercial standards and quantified by liquid scintillation counting.

Intracellular free calcium ($[Ca^{2+}]_i$) measurement. $[Ca^{2+}]_i$ was measured using the Ca^{2+}-sensitive dye fura-2 as described previously (28). Briefly, cells were rinsed with HBSS (10 mM NaCl, 4.2 mM KCl, 0.5 mM NaH_2PO_4, 0.4 mM Na_2HPO_4, 0.4 mM $MgSO_4$, 0.3 mM $MgCl_2$, 6 mM glucose, 0.1% bovine serum albumin and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, pH 7.4), and loaded with 1 μM fura-2/AM in HBSS at room temperature for one hour. Dye-loaded cells were incubated in HBSS for one hour to maximize the de-esterification of intracellular fura-2/AM. Fura-2 fluorescence was measured using a microscope-based single-cell Ca^{2+} imaging system (Intracellular Imaging, Inc., Cincinnati, OH). In some experiments, the HBSS was made nominally Ca^{2+}-free by the addition of 2.5 mM EGTA.

Calcium uptake assay. ROS 17/2.8 cells were assayed for Ca^{2+} uptake using procedures described previously (3, 4). Cells were seeded into 3.5cm dishes and grown to approximately 50% confluence. Culture medium was aspirated and the cells were washed with resting buffer (132 mM NaCl, 5 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM D-glucose and 25 mM Tris–HCl, pH 7.4). The cells then were incubated in resting buffer containing 1,25$(OH)_2D_3$, C18-ceramide, C2-ceramide or ethanol vehicle, or in high K^+ buffer (5 mM NaCl, 132 mM KCl, 1.3 mM $MgCl_2$, 1.2 mM $CaCl_2$, 10 mM D-glucose and 25 mM Tris–HCl, pH 7.4). All uptake solutions contained 12.5 μCi $^{45}Ca^{2+}$/ml. Uptake was terminated after two minutes by aspiration of the labeling solution followed immediately by three washes with ice-cold resting buffer. Cell-associated $^{45}Ca^{2+}$ was extracted for two hours in 0.5 N NaOH and measured by liquid scintillation counting.

RESULTS

1,25$(OH)_2D_3$ Induces Sphingomyelin Turnover in ROS 17/2.8 Osteosarcoma Cells

To investigate whether the intracellular levels of sphingolipid second messengers are generated in response to 1,25$(OH)_2D_3$, it was necessary to optimize the methods to detect individual products of sphingomyelin turnover. We found that the specificity and efficiency of [^{3}H]serine incorporation into sphingomyelin and total lipids was superior to metabolic labeling using [^{3}H]palmitic acid (data not shown). ROS 17/2.8 cell lipids were metabolically labeled to equilibrium with L-[3-^{3}H]serine and the levels of sphingomyelin and ceramide were measured at various times after treatment with 1,25$(OH)_2D_3$. As shown in Fig. 1, 1,25$(OH)_2D_3$ induced a time-dependent reduction of sphingomyelin. Maximal hydrolysis of sphingomyelin (73% of control) was observed 60 min after addition of 1,25$(OH)_2D_3$. The reduction in sphingomyelin 60 min after hormone treatment was accompanied by a concomitant 33% increase in ceramide levels suggesting that 1,25$(OH)_2D_3$ stimulated a sphingomyelinase activity that converts sphingomyelin to ceramide. The levels of both sphingomyelin and ceramide returned to control levels by 3.5 h.

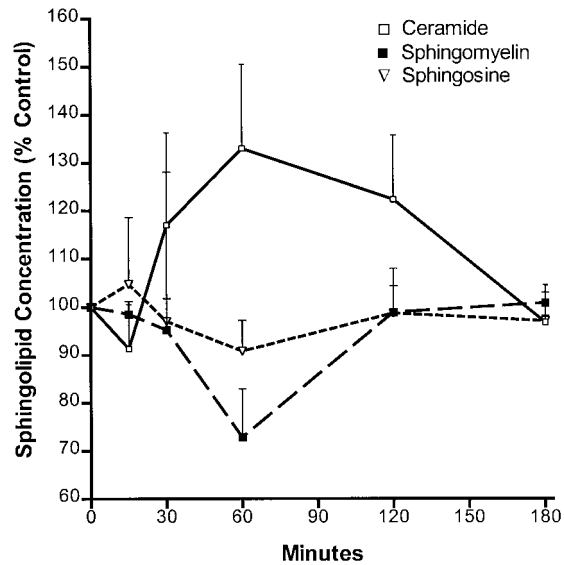

FIG. 1. Effects of 1,25$(OH)_2D_3$ on sphingomyelin, ceramide and sphingosine levels in ROS 17/2.8 osteoblastic cells. ROS 17/2.8 cells were labeled with L-[3-^{3}H]serine as described under Materials and Methods. 1,25$(OH)_2D_3$ or the same volume of ethanol vehicle was added to experimental and control groups, respectively. At the indicated times, total lipids were extracted and separated by thin-layer chromatography. The separated radiolabeled sphingomyelin, ceramide and sphingosine were quantified by liquid scintillation counting. Values for each experiment were normalized as the percentage of the control group. Data are presented as means ± standard deviation.

Effects of Sphingomyelin Metabolites on $[Ca^{2+}]_i$

We next sought to determine whether ceramide generated in response to 1,25$(OH)_2D_3$ could activate Ca^{2+} influx through VSCCs in osteosarcoma cells. Ceramide often is further metabolized to form sphingosine, a potent agonist of Ca^{2+} release from intracellular stores

in preosteoblasts (25) and a variety of other cell types (reviewed in 29). Therefore, we also evaluated the effect of this sphingolipid on $[Ca^{2+}]_i$ into ROS 17/2.8 cells.

Naturally occurring ceramide (C18-ceramide) has an amide-linked 16–18 carbon fatty acyl group, whereas C2-ceramide displays higher water solubility and is cell permeant. No change in $[Ca^{2+}]_i$ was observed in response to $1,25(OH)_2D_3$ at either 1 nM or 100 nM (Fig. 2A). Addition of 25 μM C2-ceramide also did not induce a change in $[Ca^{2+}]_i$, while subsequent addition of 25 μM sphingosine elicited a Ca^{2+} transient (Fig. 2A). A similar result was seen when cells were exposed to 25 μM C18-ceramide (Fig. 2B). That sphingosine caused release of Ca^{2+} from intracellular stores was revealed by the transient increase in $[Ca^{2+}]_i$ that occurred when cells were treated in the absence of extracellular free Ca^{2+} (prior chelation with 2.5 mM EGTA; Fig. 2C). The persistent elevation in $[Ca^{2+}]_i$ observed after sphingosine treatment in Fig. 2C is the result of Ca^{2+} influx through capacitative ion channels (30, 31) and is ablated when extracellular Ca^{2+} is chelated by EGTA.

Ceramide Generated in $1,25(OH)_2D_3$-Treated ROS 17/2.8 Cells Is Not Converted to Sphingosine

Since neither $1,25(OH)_2D_3$ nor ceramide elicited increases in ROS 17/2.8 cell $[Ca^{2+}]_i$ we determined whether ceramide generated intracellularly from sphingomyelin was converted to sphingosine. The amount of sphingosine as determined by radiolabeling was approximately tenfold lower than ceramide in the same extracts (data not shown), and no increase in the former was detected after hormone treatment (Fig. 1).

$1,25(OH)_2D_3$, but Not the Products of Sphingomyelin Turnover, Induces Rapid Ca^{2+} Influx in ROS 17/2.8 Osteoblastic Cells

The absence of a $1,25(OH)_2D_3$-induced elevation in $[Ca^{2+}]_i$ in fura 2-loaded ROS 17/2.8 cells is in contrast to previous electrophysiological and $^{45}Ca^{2+}$ tracer studies that demonstrated rapid activation ion uptake in these cells through VSCCs (3). Therefore, we measured $^{45}Ca^{2+}$ uptake into ROS 17/2.8 cell two minutes after exposure to exogenous ceramide (Fig. 3). As expected, $^{45}Ca^{2+}$ influx was induced by $1,25(OH)_2D_3$ or by high K^+ buffer which activates VSCCs via depolarization of the membrane potential. In contrast, neither C18-ceramide nor C2-ceramide induced $^{45}Ca^{2+}$ influx. Ca^{2+} uptake was stimulated approximately 2.2-fold by $1,25(OH)_2D_3$, and 2.8-fold by high K^+ buffer, but uptake of the ion in ceramide-treated cells was statistically indistinguishable from cells maintained in resting buffer.

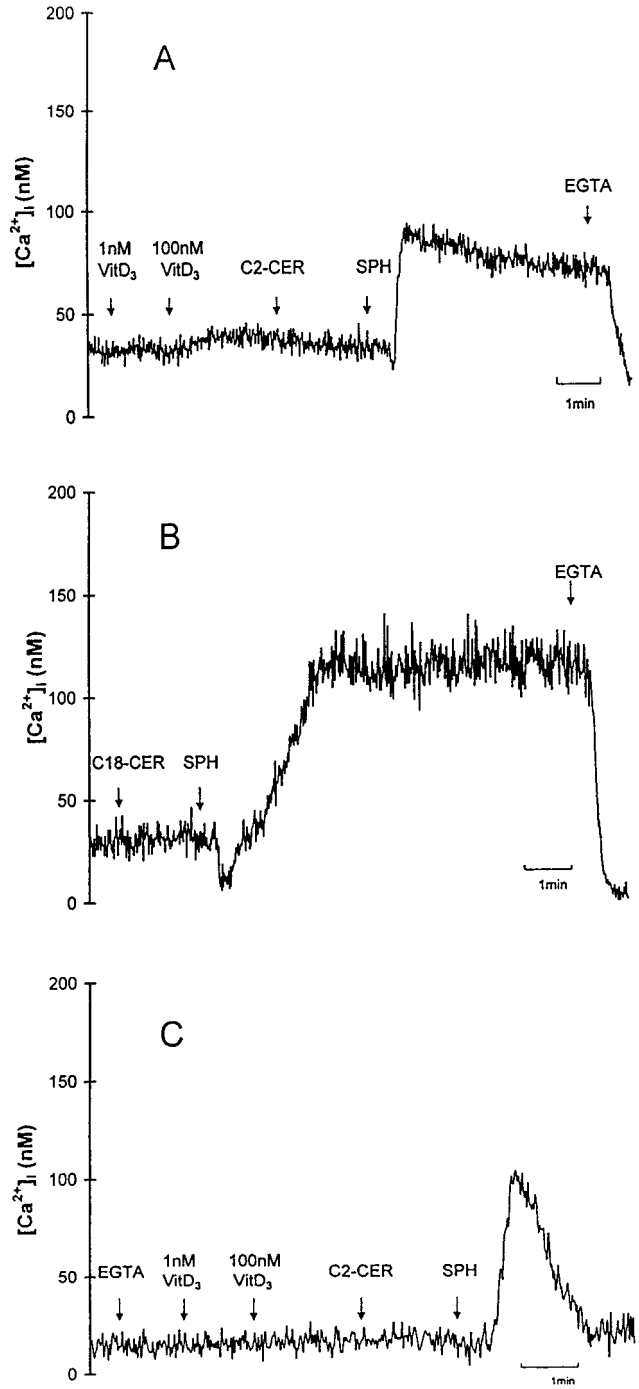

FIG. 2. Effect of $1,25(OH)_2D_3$, ceramide and sphingosine on $[Ca^{2+}]_i$ in ROS 17/2.8 cells. Measurement of $[Ca^{2+}]_i$ was made using single-cell Ca^{2+} imaging of cells loaded with the Ca^{2+}-sensitive fluorescent dye, fura 2 as described under Materials and Methods. (A) Sequential addition of 1 nM $1,25(OH)_2D_3$ ($VitD_3$), 100 nM $1,25(OH)_2D_3$, 25 μM C2-ceramide (C2-CER), 25 μM sphingosine (SPH) and 2.5 mM EGTA; (B) Sequential addition of 25 μM C18-ceramide (C18-CER), 25 μM sphingosine and 2.5 mM EGTA; (C) Sequential addition of 2.5 mM EGTA, 1 nM $1,25(OH)_2D_3$, 100 nM $1,25(OH)_2D_3$, 25 μM C2-ceramide, and 25 μM sphingosine.

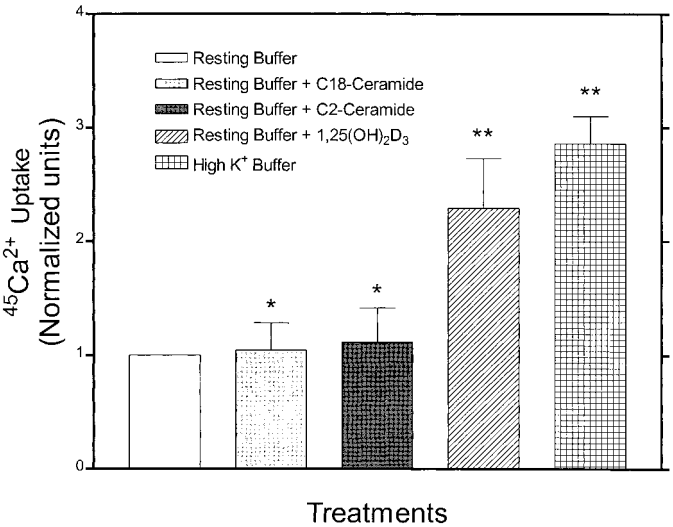

FIG. 3. $1,25(OH)_2D_3$, but not ceramide, induces Ca^{2+} uptake in ROS 17/2.8 cells. $^{45}Ca^{2+}$ uptake into ROS 17/2.8 osteoblastic cells was measured as described under Materials and Methods. $^{45}Ca^{2+}$ uptake was measured two minutes after the addition of 25 μM C18-ceramide, 25 μM C2-ceramide or 1 nM $1,25(OH)_2D_3$, or exposure of cells to high K^+ buffer to induce rapid membrane depolarization. Values for uptake within each experiment were normalized relative to that observed in resting buffer which was assigned a value of 1.0. Data represent the mean and standard deviation of five experiments in which $^{45}Ca^{2+}$ uptake was measured for two minutes. Two-tailed *t* tests were performed for the difference between the normalized value 1.0 of the resting buffer and the normalized values of the C18-ceramide, C2-ceramide, $1,25(OH)_2D_3$ or high K^+ buffer groups. $^{**}P < 0.01$; $^{*}P > 0.05$.

DISCUSSION

Sphingomyelin metabolites have been reported to transduce the effects of a number of regulatory effectors (reviewed in 32), and the data here suggest that sphingomyelin hydrolysis is involved in the intracellular signaling triggered in ROS 17/2.8 osteosarcoma cells by $1,25(OH)_2D_3$. $1,25(OH)_2D_3$ treatment elicited hydrolysis of sphingomyelin with the concomitant intracellular generation of ceramide. Cell-associated ceramide was detectable at 15 min, and maximal at 1 hour, after the addition of $1,25(OH)_2D_3$. This is similar to changes in ceramide and sphingomyelin levels observed in HL-60 cells treated with $1,25(OH)_2D_3$ (ref. 12).

Previous research has shown the kinetics of receptor-mediated ceramide formation to be complex and variable with maximum elevations ranging from seconds to hours after exposure to agonists. Our findings suggest that $1,25(OH)_2D_3$ induces sphingomyelinase activity in osteosarcoma cells within minutes of hormone treatment. Thus, elevated sphingomyelin turnover should be considered among the growing list of rapid effects of $1,25(OH)_2D_3$. However, ceramide appears not to be involved in the $1,25(OH)_2D_3$-induced activation of L-type Ca^{2+} channels in the plasma membranes of osteoblastic cells, a phenomenon that occurs in seconds to minutes (3, 4, 9, 10). Exposure of cells to exogenous ceramide did not lead to demonstrable Ca^{2+} influx, in contrast to the ion flux apparent in cells treated with $1,25(OH)_2D_3$ or incubated in high K^+ buffer to depolarize the membrane potential.

Other metabolites of sphingomyelin, such as sphingosine and sphingosine phosphate, have potential roles in signal transduction such as the inhibition of protein kinase C and the activation of Ca^{2+} release from intracellular stores (25, 33, 34). We found that the ceramide generated in ROS 17/2.8 cells in response to $1,25(OH)_2D_3$ was not metabolized to sphingosine. This is consistent with a previous analysis of HL-60 cells in which $1,25(OH)_2D_3$ did not promote formation of sphingosine from ceramide (23). While our data cannot exclude the alternative possibility that sphingosine did not accumulate but instead turned over rapidly, we detected rapid $[Ca^{2+}]_i$ elevations in ROS 17/2.8 cells incubated with exogenous sphingosine. Thus, the lack of $[Ca^{2+}]_i$ transients in cells treated with $1,25(OH)_2D_3$ or ceramide argues against any functionally significant conversion of ceramide to sphingosine.

That $1,25(OH)_2D_3$ treatment stimulated $^{45}Ca^{2+}$ influx but did not lead to detectable increases in $[Ca^{2+}]_i$ in fura 2 loaded ROS 17/2.8 cells initially was puzzling in light of our current results and previous data demonstrating the opening of VSCCs in response to this hormone (3, 4). However, we also found $[Ca^{2+}]_i$ in UMR 106 osteosarcoma cells and MC3T3-E1 pre-osteoblasts to be unaltered by $1,25(OH)_2D_3$ under conditions where addition of the hormone elicited a large $[Ca^{2+}]_i$ transient in BC_3H1 myocytes (28; Meszaros and Karin, unpublished). One explanation is that fura-2 is insufficiently sensitive to detect the Ca^{2+} influx induced by $1,25(OH)_2D_3$ in non-excitable osteoblastic cells where VSCC levels are estimated at 1500-3000 channels per cell (3). Another possibility is that the Ca^{2+} influx induced by $1,25(OH)_2D_3$ is not amplified by Ca^{2+}-induced Ca^{2+} release from intracellular stores. Consistent with the latter interpretation is the lack of release of Ca^{2+} from intracellular stores in osteosarcoma cells treated with caffeine, a potent agonist of Ca^{2+}-sensitive Ca^{2+} release channels (Meszaros and Karin, unpublished).

$1,25(OH)_2D_3$ can induce osteoblast differentiation as evidenced by the expression of the bone matrix components osteopontin (35), osteocalcin (36) and matrix Gla protein (37). Ceramide can induce cellular differentiation in other cell lines (13, 23), most likely in its role as a second messenger in activation of downstream targets such as ceramide-activated protein kinase (38), ceramide-activated protein phosphatase (39) and the protein kinase C ζ isoform (40). The data presented here suggest a link between $1,25(OH)_2D_3$ action and ceramide signaling in osteoblastic cells. Further research may reveal a role for ceramide and its down-

stream effectors in $1,25(OH)_2D_3$-induced osteoblast differentiation.

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